Clonal Characteristics in Layers of Human Atherosclerotic Plaques

A Study of the Selection Hypothesis of Monoclonality

Thomas A. Pearson, MD, John M. Dillman, BA, Kim Solez, MD, and Robert H. Heptinstall, MD

Two hypotheses have been proposed to explain the origin of monoclonal cell populations within human atherosclerotic plaques. The first of these proposes a mutational origin; the second suggests that the single clone of cells with the greatest proliferative advantage is selected following repetitive intimal injury. If the selection hypothesis is true, monoclonality should be observed more frequently in the layer of plaque most recently formed. Glucose-6-phosphate dehydrogenase (G-6-PD) isoenzymes were used as cellular markers in aortas of females heterozygous for the A and B isoenzymes. Ten plaques were divided into 45 portions, each of which was subdivided into upper layer, lower layer, and underlying media. No predominance of monoclonality was observed in the upper or lower layers of plaque, with 53% of samples from each layer being monoclonal. In all, 73% of portions of plaque contained at least one monoclonal layer. The layers tended to resemble each other in their clonal characteristics, with 60% of portions having layers with the same clonal characteristics. A significant correlation between isoenzyme distributions in upper and lower layers of the same portion was observed. No consistent trends in isoenzyme distribution in the three layers of each portion were observed. The results are interpreted as providing no evidence for clonal selection as the mechanism by which human atherosclerotic plaques become monoclonal. (Am J Pathol 93:93–102, 1978)

THE PROLIFERATION of smooth muscle cells is thought to be the principal process in the formation of human atherosclerotic fibrous plaques.[1] The clonal characteristics of fibrous plaques have been studied by Benditt and Benditt[2] and by ourselves,[3,4] providing evidence that plaques consist of monoclonal cell populations. These studies have used the X-linked enzyme glucose-6-phosphate dehydrogenase (G-6-PD) as a cell marker in black females who are heterozygous for electrophoretically separable isoenzymes A and B. G-6-PD can be used as a cell marker in the study of clonal origins since the phenomenon of X-chromosome inactivation during embryogenesis prevents the expression of one of the two X-chromosomes in each female cell and its descendent cells.[5] This X-chromosome inactivation is thought to be random and permanent, so that normal tissue will consist of a mosaic of patches of cells expressing the

From the Department of Pathology, The Johns Hopkins University School of Medicine and Hospital, Baltimore, Maryland.

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Address reprint requests to Robert H. Heptinstall, MD, Department of Pathology, The Johns Hopkins Hospital, Baltimore, MD 21205.

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same isoenzyme. However, these patches are so minute that the assay of even very small bits of normal tissue will yield both isoenzymes. Monoclonal lesions, on the other hand, will contain only one isoenzyme type in the heterozygous female.[6,7]

There are at least two hypotheses to explain the origin of these monoclonal cell populations. One hypothesis states that mutation initiates a monoclonal proliferation in a manner similar to that observed in benign neoplasms [8-10]; the second proposes that the observed monoclonal characteristics result from the selection of the single clone of cells with the greatest proliferative advantage. This selection might be caused by a) recurrent intimal injury which results in multiple cycles of cell death and proliferation, leading to the repetitive sampling of intimal cell populations and selection of the clone with greatest proliferative advantage [7,11] or b) the senescence of certain clones of arterial lining cells.[12]

This study seeks to test the hypothesis that selection occurs within human atherosclerotic fibrous plaques. If repetitive sampling and clonal selection occur as the plaque grows in thickness, then the emergence of monoclonality should be observed in those layers of the plaque most recently formed. If plaque growth occurs in cells at the intimal surface, then layers of plaque nearest the arterial lumen should contain more monoclonal characteristics than layers below them. Alternatively, if plaque growth occurs at the base of the plaque, then monoclonality should be observed more frequently in the layers of plaque nearest the media. Observation of these trends would provide evidence for selection as the mechanism by which monoclonal plaques arise.

Materials and Methods

Collection and Handling of Tissue

Aortas from black females were collected from the autopsy services of The Johns Hopkins Hospital and the Office of the Medical Examiner for the State of Maryland. The tissue was collected as soon as possible after death, wiped clean of adhering blood, placed intimal side up on a piece of cardboard, frozen in liquid nitrogen, sealed in a plastic bag, and stored at −70 C. G-6-PD heterozygosity was determined from a small piece of uninvolved aortic wall stored separately.

Before dissection of atherosclerotic lesions, the aorta was allowed to thaw and was kept cool on a bed of ice. The size and location of the plaques were documented by placing a clear plastic sheet over the aorta and tracing on the sheet outlines of the aorta, various landmarks of the aorta such as ostia of major arteries, and the plaques to be dissected. Surface areas of the plaques were later calculated by comparison of the weights of the overlay outlining the lesion with the weight of an overlay of known surface area. All the plaques included in this study were raised, were white or pearly in color, and contained a cap of dense connective tissue on the intimal side of the lesion.

Dissection and Histologic Examination of Plaques

The plaques were first dissected grossly; a No. 7 scalpel blade was used to incise around the margin of the lesion, thus isolating the lesion together with underlying media from any surrounding uninvolved media. Adventitia was then removed from the tissue. The plaque was then further divided into multiple portions if its size allowed. Each *portion* consisted of the full thickness of plaque along with underlying media and had intimal surface dimensions of approximately 3 × 3 mm. The approximate location of each portion was recorded on the transparent overlay. The portion was then divided into three *layers*. The *underlying media* layer was dissected off under a 15 to 25 power dissecting microscope; the remainder of the portion was then separated into *upper* and *lower plaque* by dividing the plaque in half along a plane parallel to the intimal surface.

Each of the layers of upper plaque, lower plaque, and underlying media was then bisected. Half was prepared for determination of G-6-PD isoenzymes by cellulose acetate electrophoresis. The other half was fixed for histology in 10% buffered formalin (pH 7.0). After embedding the tissue in paraffin, 4-μ sections were cut and stained with hematoxylin–eosin to determine whether there was contamination of plaque by underlying media. The portion was excluded from the study if contamination was present.

Cellulose Acetate Electrophoresis

The methods used in cellulose acetate electrophoresis have been presented in detail elsewhere.[9] A modified buffer consisting of 0.120 M Tris, 0.046 M borate, and 0.001 M EDTA (Sigma), pH 8.7, was used in this study. Following electrophoresis, the cellulose acetate plates were placed for 10 seconds in a solution containing 2 mg nicotinamide adenine dinucleotide phosphate (NADP), 5 mg glucose-6-phosphate, 1 mg MTT tetrazolium, and 0.3 mg phenazine methosulfate (Sigma) in 5 ml of 0.1 M Tris buffer, pH 8.0, and were developed in total darkness to proper intensity. Eight samples were applied to each cellulose acetate plate, allowing layers of upper plaque, lower plaque, and underlying media from the same portion to be assayed under identical conditions.

The relative amount of enzyme activity in each of the isoenzyme bands was determined using a densitometer (Helena Laboratories) with integration of the area under the densitometric curve. The results of electrophoresis were expressed as the percentage of total enzyme activity in the B isoenzyme band (%B isoenzyme).

Definition of Monoclonality and Statistical Analysis

The results of electrophoresis were used to classify the layers of plaque as monoclonal or not monoclonal. The mean percentage of the total enzyme activity in the B isoenzyme band of 8 to 12 samples of media for each aorta was calculated. Around that mean were placed ± 3 standard deviation (99.7%) confidence limits. Those layers whose isoenzyme values fell within these confidence limits were considered polyclonal. Layers of plaque whose percentage of total isoenzyme in the B band fell outside these confidence limits were considered monoclonal.

The results of electrophoresis were analyzed by grouping the portions according to the clonal characteristics of their layers and according to the gradation of %B isoenzyme observed between media, lower plaque, and upper plaque. The χ^2 test with Yates' correction for continuity was used to determine if the number of portions in the various groups were distributed in a manner significantly different than that expected by chance.[13] The relationship of %B isoenzyme values between layers of plaque was examined by calculation of correlation coefficients, r.[13]

Results

G-6-PD isoenzyme patterns (%B isoenzyme) were determined on 45 portions from 10 plaques dissected from 6 patients' aortas (Table 1). The surface area of the plaques ranged from 51.2 to 254.7 sq mm. Each portion consisted of three layers: upper plaque, lower plaque, and underlying media. Every plaque in the study contained at least one monoclonal layer. The isoenzyme type predominating in monoclonal layers of plaque was evenly divided between A and B (upper 15A, 9B; lower: 14A, 10B). Several plaques contained monoclonal layers of both the A and B isoenzyme types. Of the 45 portions, 33 (73.3%) contained at least one monoclonal layer (Table 2).

The results were examined to determine if upper layers of plaque were more frequently or less frequently monoclonal than lower layers of plaque. If clonal selection occurred with increasing plaque thickness, a predominance of monoclonal portions would be expected in one or the other layer. Of the 45 upper layers, 24 (53.3%) had monoclonal characteristics, and identical numbers of lower layers had monoclonal characteristics (Tables 1 and 2). Thus, the results show no pattern consistent with selection favoring either the upper or the lower layers of plaque. The distribution of portions was not significantly different ($\chi^2 = 1.5$, 1 d.f., N.S.) from that of random distribution in the four categories grouped according to the clonal characteristics of the layers within each portion (Table 2). However, the upper and lower layers of plaque appeared to exhibit similar clonal characteristics (both or neither monoclonal) in the majority of portions (27/45, 60%).

The resemblance of clonal characteristics in upper and lower layers

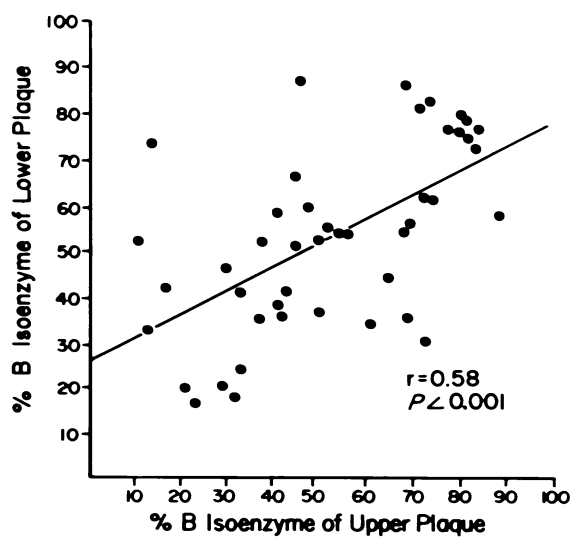
Table 1—Clonal Characteristics of Layers of Plaques From Heterozygous Females

Patient	Plaque No.	Plaque surface area (sq mm)	No. of portions	No. of monoclonal layers (No. of A; No. of B)	
				Upper	Lower
1	1	186.2	12	9 (9A)	7 (5A, 2B)
2	1	254.7	9	3 (3A)	3 (2A, 1B)
3	1	81.2	1	1 (1A)	1 (1A)
4	1	120.3	2	2 (2A)	2 (2A)
5	1	64.5	5	2 (2B)	0
	2	51.2	3	0	2 (2A)
6	1	55.3	2	0	1 (1A)
	2	54.0	3	1 (1B)	2 (1A, 1B)
	3	72.9	3	2 (2B)	2 (2B)
	4	121.2	5	4 (4B)	4 (4B)
Total	10		45	24 (15A, 9B)	24 (14A, 10B)

Table 2—Number of Portions of Plaques Containing Monoclonal Layers

Characteristic of lower layer	Characteristic of upper layer					
	Monoclonal		Not monoclonal		Total	
	n	%	n	%	n	%
Monoclonal	15	33	9	20	24	53
Not monoclonal	9	20	12	27	21	47
Total	24	53	21	47	45	100

from the same portion was studied further by plotting the %B isoenzyme in lower layers of plaque vs the %B isoenzyme in the same portion's upper layer of plaque (Text-figure 1). A relatively strong, statistically significant correlation ($r = 0.58$, $P < 0.001$) was observed between isoenzyme patterns in upper and lower plaque. Although most portions fell along the regression line, a few portions did show marked differences in %B isoenzyme observed in upper vs lower layers of plaque. The results in Text-figure 1 are in contrast to the weaker but significant correlation between lower layers of plaque and underlying media ($r = 0.42$, $P<0.01$) and the absence of correlation of %B isoenzyme between upper layers of plaque and underlying media ($r = 0.23$, N.S.). These results show that upper and lower layers of plaque tend to resemble each other in %B isoenzyme distribution to a greater extent than they resemble the media lying beneath them.



TEXT-FIGURE 1—The relationship between G-6-PD isoenzyme values within each portion of plaque is examined by plotting the %B isoenzyme value of lower plaque vs the %B isoenzyme value of upper plaque for each portion of plaque.

Even in the absence of a predominance of monoclonal characteristics in either layer of plaque, the demonstration of gradations in isoenzyme distribution could still provide evidence for the presence of clonal selection in plaques. Since each portion contains three layers (upper plaque, lower plaque, and underlying media), trends in %B isoenzyme in the layers in each portion could be sought. Some of the trends in %B isoenzyme observed are illustrated in Text-figure 2, which is a schematic diagram of four portions taken from a cross-section of the plaque from Case 1. The %B isoenzyme values in samples of underyling media varied little and clustered around the mean of samples of media from that aorta. In contrast, great variation was observed in upper and lower layers of plaque, with layers falling both above and below the 99.7% confidence limits for samples of media (thus having monoclonal A and monoclonal B layers adjacent to each other). The presence of trends in isoenzyme distribution was sought within each portion. Portions I and II in Text-figure 2 are examples of patterns consistent with clonal selection in upper layers of plaque, ie, a gradation of isoenzyme distribution occurs from media through lower plaque to the extreme in upper plaque; in this case the gradation is one with decreasing %B isoenzyme. Portions III and IV in Text-figure 2 show trends consistent with the occurrence of clonal selection in lower layers of plaque. In portion III, the %B isoenzyme in lower plaque is greater than that of either the upper plaque or the underlying media. In portion IV, the %B isoenzyme in the lower plaque is less than that in either the upper plaque or the media underlying it. In the 45 portions, isoenzyme trends consistent with clonal selection in upper layers were observed in 19 (42.2%), whereas trends consistent with clonal selection in lower layers were observed in the majority of portions (26/45, 57.8%) (Table 3). The distribution of the portions into the four possible

G-6-PD ISOENZYMES IN LAYERS OF FIBROUS PLAQUE AND UNDERLYING MEDIA

	I	II	III	IV
Upper Plaque	30.4	16.9	45.5	32.0
Lower Plaque	46.4	41.7	87.2	17.8
Underlying Media	53.7	50.5	58.7	63.4

Mean % B Isoenzyme in Portions of Underlying Media = 59.3
99.7 % Confidence Limits = 41.6 - 77.0

TEXT-FIGURE 2—Electrophoresis results from an actual cross-section from Patient 1 are illustrated diagrammatically. The %B isoenzyme values are shown in each of the three layers from four portions, numbered I, II, III, and IV. The mean of portions of media and the 99.7% confidence limits around the mean for that aorta are shown below the chart. It is noted that five layers (4A, 1B) in upper and lower plaques fall outside these confidence limits, indicating monoclonality.

Table 3—G-6-PD Isoenzyme Patterns as Evidence of Clonal Selection in Layers of Fibrous Plaques

	Pattern of %B isoenzyme within layers of plaque	No. of portions
Consistent with clonal selection in upper layer	Upper > Lower > Underlying media	10
	Upper < Lower < Underlying media	9
		Total 19 (42%)
Consistent with clonal selection in lower layer	Upper > Lower < Underlying media	8
	Upper < Lower > Underlying media	18
		Total 26 (58%)

trend groups was not significantly different from that expected at random ($\chi^2 = 4.47$, 3 d.f., N.S.).

Discussion

The monoclonal nature of atherosclerotic fibrous plaques has been demonstrated by independent investigators with unequivocal results.[2-4] Although the laboratory observations have been firmly established, a variety of interpretations have been made as to the mechanism by which monoclonal cell populations arise. In the mutation hypothesis, as proposed by Benditt,[2,8-10] it is stated that three stages occur in atherogenesis: 1) initiation with mutation occurring in a smooth muscle cell, 2) the promotion of the proliferative advantage of the mutant cell by action of a variety of agents, and 3) the complication of the plaque with degeneration of cells within the lesion. The mutation hypothesis has strong implications regarding the mechanism of action of risk factors such as cigarette smoking, hypercholesterolemia, and hypertension.

An alternative hypothesis states that monoclonal cell populations arise following the selection of the clone with the greatest proliferative advantage. The hypothesis has been developed along the following lines: Injury to the endothelial lining of the artery initiates proliferation by exposing subintimal cells to a variety of proliferative stimuli.[1] It has been suggested that the recurrent injury of these intimal cell populations causes cycles of cell death and proliferation.[7] Fibrous plaques are known to have a layered appearance when examined microscopically and it is conceivable that these layers of smooth muscle cells and connective tissue are the products of these cycles of cell proliferation. It is proposed that as these cycles continue, multiple samplings of intimal cell populations occur, leading to the selection of the cell population which has the greatest proliferative advantage.

There have been at least two mechanisms proposed to account for the proliferative advantages assumed by certain clones of cells within athero-

sclerotic plaques. First, heterogeneity in proliferative capacity within clones of cells is thought to be due to the proclivity of some clones to become senescent, while other clones continue to proliferate.[12] The senescent clones may then lose their ability to inhibit proliferation in neighboring clones, leading to the continued growth and accumulation of proliferating clones of cells.[1] Considerable variability in the proliferative capacity of different clones of cells has been observed in human fibroblasts *in vitro* [12] and in arterial smooth muscle cells from hypercholesterolemic swine *in vivo*.[11] Senescence of clonal cell populations has been observed more frequently in humans with conditions associated with the formation of atherosclerotic plaques, eg, advancing age and certain degenerative diseases.[14] Clonal senescence occurs more frequently in cells from the abdominal aorta than from the thoracic aorta,[15] correlating with the topographic distribution of human atherosclerotic lesions. The second mechanism proposed to account for the proliferative advantage of certain clones of cells is that the expression of certain genes on one of the X-chromosomes in the human female gives the cell a selective advantage over other cells in which the X-chromosome had been inactivated.[11] Thus, only cells expressing genes on the same X-chromosome would proliferate and only a single isoenzyme of G-6-PD would be expressed.

The results of this study provide no evidence for clonal selection occurring during plaque growth. Although the results confirm previous observations of monoclonality within atherosclerotic plaques, the examination of layers of plaques failed to show a predominance of monoclonality in either upper or lower layers. Isoenzyme patterns within upper plaque, lower plaque, and underlying media did not show trends consistent with the presence of clonal selection in upper or lower layers of plaque. There was also no evidence for genes on a single X-chromosome conveying a selective advantage, since several aortas contained plaques with both monoclonal A and monoclonal B cell populations. In fact, both monoclonal A and monoclonal B layers were found in the same plaque in several instances. Rather than showing patterns consistent with selection in one of the layers, this study's results show that upper and lower layers are similar in their clonal characteristics.

Several interpretations of these data are possible. First, it is possible that clonal selection actually does occur in plaques but that the selection occurs early in plaque development when the plaques are small and contain relatively few cells. Therefore, this study of large plaques might not detect clonal selection which had occurred when the plaques were much smaller. In previous studies of both small and large plaques,[3,16] we were not able to show any marked trends in clonal characteristics as

plaques grow. Second, it is possible that clonal selection does not occur as the plaque increases in thickness; rather, selection may occur with circumferential growth of plaque cells. This possibility has yet to be investigated. Finally, it is possible that clonal selection following repetitive intimal injury plays no role in plaque formation. In studies of G-6-PD isoenzyme patterns within cutaneous scars (lesions known to result from tissue injury) we have observed neither monoclonal characteristics nor evidence for clonal selection. In fact, the only direct *in vivo* evidence for the occurrence of clonal selection in humans has been derived from the study of neoplasms.[17] Mutation is thought to occur initially within neoplasms in a number of clones of cells, with clonal selection being important in the evolution of these tumor cell populations.[18] Thus, even if clonal selection were demonstrated in plaque formation, mutation might still be the event initiating cell proliferation. Further studies are needed to characterize the origin and growth of monoclonal cell populations within human atherosclerotic plaques.

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